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(54) Title: RECOMBINANT HYBRID PORIN EPITOPES

(57) Abstract

A polypeptide that is non-toxic to E. coli wherein the polypeptide comprises at least one antigenic sequence present in P.IA of *N. gonorrhoeae* and at least one antigenic sequence present in P.IB of *N. gonorrhoeae*.

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RECOMBINANT HYBRID PORIN EPITOPES

The diseases caused by the gonococcus Neisseria gonorrhoeae, such as gonorrhea, are among the most prevalent venereal diseases in the world. Such diseases have proven to be difficult to control by traditional antibiotic and vaccine treatments.

In PCT application WO 89/04873, Carbonetti and Sparling describe an approach to vaccines based on a porin protein present in the outer membrane of N. gonorrhoeae. This protein, which is called protein I, forms pores that allow small hydrophilic solutes to pass through the outer membrane. Protein I (P.I) may be divided into two genetically and immunologically distinct serovar groups present in N. gonorrhoeae, P.IA and P.IB.

The DNA sequence of the P.I gene of FA19, a IA serovar, is shown as Figure 3 of WO 89/04873. The DNA sequence of the PI gene of MS11, a IB serovar, is shown as Figure 9 of WO 89/04873. The DNA sequences shown in Figures 3 and 9 as well as the corresponding amino acid sequences, which are also shown, are incorporated herein by reference. The DNA sequence of the PI gene of R10, a IB serovar, and the corresponding amino acid sequence is disclosed by Gotschlich et al. in Proc. Nat'l Acad. Sci. USA 84, 8135-8139 (1987). The DNA and amino acid sequences of P.IB as reported by Gotschlich et al. are incorporated herein by reference.

Successful approaches to the prevention, detection and treatment of gonococcal infection must be directed to both of the clinically important N. gonorrhoeae serovar groups. One approach to solving this problem is the

development of intertypic hybrids. Such hybrids are generally prepared by inserting a selectable marker into the DNA of a strain of one serovar, and transfecting the DNA into a strain of the other serovar. Random recombination of the largely homologous P.I genes in the transfected cell leads to a hybrid gene that expresses some epitopes of both P.IA and P.IB. Such intertypic hybrids have been described by Carbonetti and Sparling in PCT application WO 89/04873 and by Shinners and Catlin in J. Infect. Dis. 158, 529-536 (1988).

One difficulty with the intertypic hybrid approach is that the recombinant porin proteins are full length P.I proteins, which have approximately 300 amino acids. Such proteins are difficult to work with due to poor solubility, and to be produced by genetic engineering methods in *E. coli* due to toxicity problems. The difficulty of growing large numbers of porin gene-containing bacterial cells has been described by Gotschlich et al in Proc. Nat'l Acad. Sci USA 84, 8135-8139)1987) and Carbonetti and Sparling, PCT application WO 89/04873.

Moreover, intertypic hybrids result from random recombination events, and do not constitute a rational approach to the design of a protein useful in diagnostic methods, vaccines and treatments of diseases caused by *N. gonorrhoeae*. One hopes to do better than to have to pick and choose among various randomly formed proteins to determine which, if any, might be useful.

There is a need, therefore, for rationally designed chimeric proteins that contain epitopes of both P.IA and P.IB. There is a particular need for such chimeric protein that are not toxic to *E. coli*, and that have fewer

than 300 amino acids.

SUMMARY OF THE INVENTION

These and other objectives as will be apparent to those having ordinary skill in the art have been met by providing a polypeptide that is non-toxic in E. coli. The polypeptide comprises at least one antigenic sequence present in P.IA of N. gonorrhoeae and at least one antigenic sequence present in P.IB of N. gonorrhoeae.

The invention further relates to a polypeptide comprising at least one antigenic sequence present in P.IA of N. gonorrhoeae and at least one antigenic sequence present in P.IB of N. gonorrhoeae. The total number of amino acids in the antigenic sequence present in P.IA is no more than 125. The total number of amino acids in the antigenic sequence present in P.IB is also no more than 125.

DESCRIPTION OF THE FIGURES

Figure 1A shows the antigenic sequences corresponding to P.I fragments 1-6. Each fragment optionally contains an additional N-terminal cysteine residue. The amino acid numbers correspond to the amino acid residues of P.IA from N. gonorrhoeae strain FA19 (fragments 1-4) or of P.IB from N. gonorrhoeae strain MS11 (fragments 5 and 6).

Figure 1B shows the relative positions of fragments 1-4 on P.IA from N. gonorrhoeae strain FA19 and fragments 5 and 6 on P.IB from N. gonorrhoeae strain MS11.

Figure 2 shows the nucleotide sequence of the polylinker in PATH20.

Figure 3A shows the nucleotide sequence of the six oligonucleotides (a-f) used to prepare a chimeric polypeptide containing fragments 2 and 6 called GC26 (see Example 1).

Figure 3B shows the relationship of oligonucleotides a-f to each other and to the PATH20 restriction sites EcoRI and HindIII in pGC26.

Figure 4A shows the nucleotide sequence of the four oligonucleotides (g-j) used to prepare a chimeric polypeptide containing fragments 2, 6, and 4 called GC264.

Figure 4B shows the relationship of oligonucleotides g-j to each other and to the BsmI and HindIII sites of pGC26.

Figure 5 shows the nucleotide sequence used to express GC26 in bacteria (see Example 1).

DETAILED DESCRIPTION

Fragments

The invention relates to chimeric polypeptides comprising fragments containing antigenic sequences of both P.IA and P.IB from an *N. gonorrhoeae* strain of a IA or IB serovar, respectively. Strains of both serovar groups IA and IB are known. Some known IA strains include, for example, FA19, FA6599, FA6642, NRL V.15, NRL 7929, and NRL G.7. Some strains of the IB serovar include, for example, MS11, R10, NRL T.13, NRL1955, NRL5767, 4403(Pgh3-2), 4408(Pgh3-1), and 4409(51288).

Fragments containing antigenic sequences of P.IA and

P.IB may be selected on the basis of generally accepted criteria of potential antigenicity and/or exposure. Such criteria include the hydrophilicity and relative antigenic index, as determined by surface exposure analysis of P.IA and P.IB proteins. The determination of appropriate criteria is known to those skilled in the art, and has been described, for example, by Hopp et al, Proc. Nat'l Acad. Sci. USA 78, 3824-3828 (1981); Kyte et al, J. Mol. Biol. 157, 105-132 (1982); Emini, J. Virol. 55, 836-839 (1985); Jameson et al, CA BIOS 4, 181-186 (1988); and Karplus et al, Naturwissenschaften 72, 212-213 (1985). Amino acid domains predicted by these criteria to be surface exposed are selected preferentially over domains predicted to be more hydrophobic or hidden.

Fragments 1-6, which are shown in Figure 1A, are suitable antigenic sequences. Fragments 1-4 contain amino acid sequences found in P.IA of gonococcal strain FA19. Fragments 5 and 6 contain amino acid sequences found in P.IB of gonococcal strain MS11. These fragments were disclosed in a simultaneously-filed continuation-in-part of a U.S. patent application of Carbonetti and Sparling having serial number 07/242,758, which is equivalent to WO 89/04873.

Chimeric polypeptides

The chimeric polypeptides of the present invention comprise at least two fragments. At least one fragment comprises an antigenic sequence present in P.IA of *N. gonorrhoeae*. At least one other fragment comprises an antigenic sequence present in P.IB of *N. gonorrhoeae*.

In addition, the chimeric polypeptides of the

invention satisfy certain immunological criteria. First, the polypeptides bind to monoclonal and polyclonal sera specific to type IA and IB porin serovars of N. gonorrhoeae, as well as to monoclonal and polyclonal antibodies specific to each individual fragment of the chimeric polypeptide. In addition, the chimeric polypeptides illicit specific serum antibody responses in mammals, including humans, toward at least one epitope of both a type A and a type B serovar.

Any rationally designed chimeric polypeptide that has at least two antigenic sequences, at least one of which is present in P.IA and at least one other of which is present in P.IB of N. gonorrhoeae, and that satisfies the immunological criteria described above, may be used in the invention. As used in this specification, the term "chimeric polypeptide" means a polypeptide that has a rationally designed amino acid sequence, as opposed to the sequences of the randomly formed intertypic hybrids of the prior art.

In a preferred embodiment of the invention, the chimeric polypeptide comprises at least one polypeptide fragment selected from P.IA fragments 1-4 joined to at least one polypeptide fragment selected from P.IB fragments 5 and 6. The order in which the fragments occur in the polypeptide is not critical, as long as at least one P.IA fragment and at least one P.IB fragment remains antigenic. Some examples of chimeric polypeptides in accordance with the invention comprise fragments 2-6, 6-2, 4-5, 5-4, 1-6, 6-1, 2-6-4, 2-4-6, and 2-5-6. Chimers formed by fragments 2 and 6 and by fragments 2, 4, and 6 are preferred.

The fragments of the invention, such as fragments 1-

6, are preferably highly hydrophilic and, therefore, predictably immunologically valid. Each fragment comprises at least one P.I epitope. Preferably, each fragment contains more than one P.I epitope.

The antigenic fragments of the chimeric polypeptide may be sub-fragments of fragments 1-6, and contain less than all of the epitopes of its respective fragment. The fragment may have as few as one epitope. For example, a known epitope of fragment 6 is YSIPS.

Alternatively, some or all of the antigenic P.IA and/or P.IB sequences are not those represented by fragments 1-6. These other fragments may or may not overlap fragments 1-6.

The fragments of the chimeric polypeptide may contain additional amino acid sequences at either their N- or C-terminal or at both termini. These additional amino acid sequences may be present in P.IA or P.IB. Alternatively, the additional amino acid residues may be derived from proteins other than P.IA or P.IB. Such additional amino acid residues may aid in the isolation and purification of the polypeptide, aid in the presentation of the antigenic sequences to a host, or otherwise enhance the immunological properties of the polypeptides.

The additional amino acids are preferably separated from the P.IA/P.IB antigenic fragments by a suitable cleavage site. Both chemical and enzymatic cleavable sites are known in the art. Suitable examples of sites that are cleavable enzymatically include sites that are specifically recognized and cleaved by collagenase (Keil et al., FEBS Letters 56, 292-296 (1975)); enterokinase (Hopp et al., Biotechnology 6, 1204-1210 (1988)); factor

Xa (Nagai et al., Methods Enzymol. 153, 461-481 (1987)); and thrombin (Eaton et al., Biochemistry 25, 505 (1986)). Collagenase cleaves between proline and X in the sequence Pro-X-Gly-Pro wherein X is a neutral amino acid. Enterokinase cleaves after lysine in the sequence Asp-Asp-Asp-Asp-Lys. Factor Xa cleaves after arginine in the sequence Ile-Glu-Gly-Arg. Thrombin cleaves between arginine and glycine in the sequence Arg-Gly-Ser-Pro.

Specific chemical cleavage agents are also known. For example, cyanogen bromide cleaves at methionine residues in proteins.

The chimeric polypeptide should be as short as possible. Unnecessary amino acids add to the length of the polypeptide, and to the difficulty of working with it. Therefore, although additional amino acid sequences may be present at the N-terminal or C-terminal end of a fragment or between the fragments, the chimeric polypeptide preferably contains no additional amino acid sequences other than the antigenic fragments from a P.I protein, such as fragments 1-6.

The fragments are preferably identical to sequences in a strain of N. gonorrhoeae. It is, however, possible to create an equivalent fragment by deleting amino acids from a fragment without affecting all or any of the epitopes.

As is also known, it is possible to substitute amino acids in a sequence with equivalent amino acids. Groups of amino acids known normally to be equivalent are:

(a) Ala(A) Ser(S) Thr(T) Pro(P) Gly(G);
(b) Asn(N) Asp(D) Glu(E) Gln(Q);

(c) His(H) Arg(R) Lys(K);
(d) Met(M) Leu(L) Ile(I) Val(V); and
(e) Phe(F) Tyr(Y) Trp(W).

Substitutions, additions and/or deletions in the antigenic sequences may be made as long as the chimeric polypeptide of the invention continues to satisfy the immunological criteria described above. An amino acid sequence that is substantially the same as another sequence, but that differs from the other sequence by means of one or more substitutions, additions and/or deletions is considered to be an equivalent sequence. Preferably, less than 25%, more preferably less than 10%, of the number of amino acid residues in a P.IA or P.IB sequence are substituted for, added to, or deleted from the fragments in the chimeric polypeptides of the invention.

By limiting the number of amino acids, the solubility and ease of handling of the chimeric polypeptides are increased. Preferably, the total number of amino acids in the P.IA antigenic sequence or sequences, or equivalent sequence or sequences, in the chimeric polypeptide is no more than about 125, preferably no more than about 75, and more preferably no more than about 50. Similarly, the total number of amino acids in the P.IB antigenic sequence or sequences, or equivalent sequence or sequences, in the chimeric polypeptide is also no more than about 125, preferably no more than about 75, and more preferably no more than about 50.

Preferably, the chimeric porin polypeptides of the invention, unlike the P.IA, P.IB and P.IA/B intertypic hybrids of the prior art, are non-toxic in _E. coli_. Toxic proteins do not generally allow binary growth of the

organism to high absorbance at 650nm or to high cell number. Absorbance at 650nm, a reflection of total cell number and therefore cell mass, should continue to rise exponentially throughout the growth period until media exhaustion and plateau growth. Chemostatic or media supplemented growth should occur throughout the entire incubation growth phase without cell lysis or death. Cellular product, i.e., fusion protein, should accumulate steadily following induction (i.e., IAA, IPTG, 42°C, etc.) and allow product to be gathered after overnight shake culture growth. Typical 650nm absorbance values of 5-10 units are obtained in shake flasks or 25-100 units in media supplemented chemostat environments. There should be no inflection of the growth curve representing premature cell lysis during the growth cycle. The failure to achieve sufficiently high cell mass or A^{650nm} absorbance precludes economically efficient industrial scale production.

To be considered non-toxic in E. coli means that the protein is non-lytic in E. coli when it is expressed under normal conditions. To be expressed under normal conditions means that unusual steps are not taken in order to be able to express toxic proteins in E. coli.

Unusual steps include the use of highly individual and transient systems such as the T7 promoter/polymerase system of Studier (Studier and Moffatt in J. Mol. Biol. 189, 113-130 (1986) and Moffatt and Studier, Cell 49, 221-227 (1987)), and the use of specific and generally unavailable E.coli host cells (eg. BL21 pLys, etc.) or expression plasmids. Unusual methods may require the halting of growth and gathering of cell mass at relatively low A^{650} levels in order to avoid premature cell lysis and

death with the possibility of product destruction.

Normal conditions for expression of a protein in E.coli include the use of standard vector systems or generally available promoter cassettes (Trp, Tac, Trc, lambda P, beta-gal, etc.) in standard media components and during continuous growth to high cell mass and/or A^{650} values. Cell growth should occur continuously with media replacement or chemostatic growth with no premature lysis, increase in lysis-generated viscosity, or sudden drop in A^{650} upon continuous post-induction shaker or fermenter growth. Subsequent plasmid stability should remain high upon continuous growth and polypeptide production.

Synthesis of Chimeric Polypeptides

The chimeric fragments may be synthesized from individual amino acid residues by methods known in the art. Some suitable methods are described by Stuart and Young in "Solid Phase Peptide Synthesis," Second Edition, Pierce Chemical Company (1984).

The proteins of the present invention are preferably produced by means of recombinant DNA technology. General methods for producing recombinant proteins from isolated DNA are described by Sambrook et al., in "Molecular Cloning," Second ed. Cold Spring Harbor Press (1987).

Briefly, DNA coding for the desired amino acid sequence of the present invention may be obtained as fragments from natural sources and, optionally, modified. The DNA may also be synthesized in whole or in part by methods known in the art. Such methods include those described by Caruthers in Science 230, 281-285 (1985).

The DNA encoding the desired polypeptide of the invention may be replicated using a wide variety of vectors in a wide variety of host cells. The host cells may be prokaryotic or eukaryotic. The vector may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic vectors include plasmids from E. coli such as colE1, pCR1, pBR322, pUC, pKSM, pMB9, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as NM 989, M13 and other filamentous single-stranded DNA phages.

Vectors for expressing proteins in bacteria, especially E.coli, are also known. Such vectors include the PATH vectors described by Dieckmann and Tzagoloff in J. Biol. Chem. 260, 1513-1520 (1985). These vectors contain DNA sequences that encode anthranilate synthetase (TrpE) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (pEX) lambda P_L; maltose binding protein (pMAL); glutathione S-transferase (pGST) - see Gene 67, 31 (1988) and Peptide Research 3, 167 (1990).

Vectors useful in yeast are also available. A suitable example is the 2u plasmid.

Suitable vectors for use in mammalian cells are also known. Such vectors include well-known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences and vectors derived from combination of plasmids and phage DNA.

Further eukaryotic expression vectors are known in the art. See, for example, P.J. Southern and P. Berg, J.

Mol. Appl. Genet. 1, 327-341 (1982); S. Subramani et al, Mol. Cell. Biol. 1, 854-864 (1981); R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene," J. Mol. Biol. 159, 601-621 (1982); R.J. Kaufmann and P.A. Sharp, Mol. Cell. Biol. 159, 601-664 (1982); S.I. Scahill et al, "Expression And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," Proc. Natl. Acad. Sci. USA 80, 4654-4659 (1983); G. Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci. USA 77, 4216-4220, (1980).

Useful expression hosts include well-known prokaryotic and eukaryotic hosts. Some suitable prokaryotic hosts include, for example, E. coli, such as E. coli SG-936, E. coli HB 101, E. coli W3110, E. coli X1776, E. coli X2282, E. coli DHI, and E. coli MRCl, Pseudomonas, Bacillus, such as Bacillus subtilis, and Streptomyces. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the desired DNA sequence. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid

phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters or SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

The chimeric polypeptides may be purified by methods known in the art. Preferably, the polypeptides are isolated in an essentially pure state. A polypeptide is considered to be essentially pure if it is at least 85%, preferably at least 95%, and more preferably at least 99% pure. In one purification method, the chimeric polypeptide may be expressed in the form of a fusion protein with an appropriate fusion partner to facilitate purification and identification. Some useful fusion partners include maltose binding protein, Guan et al., Gene 67, 21-30 (1987); Maina et al., Gene 74, 36-373 (1988); Riggs, P., in Ausebel, F.M. et al (eds) Current Protocols in Molecular Biology, Greene Associates/Wiley Interscience, New York (1990); beta-galactosidase (Gray, et al., Proc. Natl. Acad. Sci. USA 79, 6598 (1982)); trpE (Itakura et al., Science 198, 1056 (1977)) protein A (Uhlen et al., Gene 23 369 (1983)) and glutathione S-transferase (Johnson, Nature 338, 585 (1989); and Van Etten et al., Cell 58, 669 (1989)).

Such fusion proteins may be purified by affinity chromatography using reagents that bind to the fusion partner. The reagent may be a specific ligand of the fusion partner or an antibody, preferably a monoclonal antibody. For example, fusion proteins containing beta-galactosidase may be purified by affinity chromatography using an anti-beta-galactosidase antibody column (Ullman,

Gene. 29, 27-31 (1984)). Similarly, fusion proteins containing maltose binding protein may be purified by affinity chromatography using a column containing maltose.

Optionally, the DNA that encodes the fusion protein is engineered so that the fusion protein contains a cleavable site between the chimeric polypeptide and the fusion partner. Both chemical and enzymatic cleavable sites are known in the art, as described above. Such sites allow ultimate cleavage of the fragment of the invention from its fusion partner.

In an alternative preparation method, the chimeric polypeptide is overexpressed behind an inducible promoter and purified by affinity chromatography using specific anti-chimeric polypeptide antibodies. For example, the monoclonal antibody SM101 is believed to bind to the amino terminus of P.IA that corresponds to fragment 2. SM101 is described in Virji et al., Journal of General Microbiology 133, 2639-2646 (1987). Similarly, monoclonal antibody SM24 is thought to bind the region of P.IB that corresponds to fragment 6. SM24 is described in Heckels et al., Journal of General Microbiology 135, 2269-2276 (1989).

As another alternative, the overexpressed polypeptide may be purified using a combination of ion-exchange, size-exclusion, and hydrophobic interaction chromatography using methods known in the art. These and other suitable methods are described by Marston, "The Purification of Eukaryotic Polypeptides Expressed in *E. coli*" in DNA Cloning, D. M. Glover, Ed., Volume III, IRL Press Ltd., England, 1987.

Use of chimeric polypeptides as probes

The chimeric polypeptides of the invention are useful in detecting and preventing diseases caused by gonococcal infection. For example, the proteins may be labelled and used as probes in standard immunoassays to detect antibodies against the proteins in samples, such as in the sera or other bodily fluids of patients being tested for gonorrhea. In general, a chimeric polypeptide is incubated with the sample suspected of containing antibodies to P.IA or P.IB. The polypeptide is labelled either before, during, or after incubation. Detection of labelled polypeptide bound to an antibody in the sample indicates the presence of the antibody. The antibody is preferably immobilized.

Suitable assays for detecting antibodies with polypeptides are known in the art, such as the standard ELISA protocol described by R.H. Kenneth, "Enzyme-Linked Antibody Assay with Cells Attached to Polyvinyl Chloride Plates" in Kennett et al, Monoclonal Antibodies, Plenum Press, N.Y., page 376 (1981). Briefly, plates are coated with a sufficient amount of an antigenic polypeptide to bind detectable amounts of the antibody. After incubating the plates with the polypeptide, the plates are blocked with a suitable blocking agent, such as, for example, 10% normal goat serum. The sample, such as patient sera, is added and titered to determine the endpoint. Positive and negative controls are added simultaneously to quantitate the amount of relevant antibody present in the unknown samples. Following incubation, the samples are probed with goat anti-human Ig conjugated to a suitable label. The presence of anti-polypeptide antibodies in the sample is indicated by the presence of bound label.

For use in immunoassays, the polypeptide or another molecular probe is labelled with radioactive or non-radioactive atoms or molecules. Such labels and methods for conjugating them to proteins are known in the art.

Some examples of useful radioactive labels include ^{32}P, ^{125}I, ^{131}I, and ^{3}H. Use of radioactive labels have been described in U.K. 2,034,323, U.S. 4,358,535, and U.S. 4,302,204.

Some examples of non-radioactive labels include enzymes, chromophors, atoms and molecules detectable by electron microscopy, and metal ions detectable by their magnetic properties.

Some useful enzymatic labels include enzymes that cause a detectable change in a substrate. Some useful enzymes and their substrates include, for example, horseradish peroxidase (pyrogallol and o-phenylenediamine), beta-galactosidase (fluorescein beta-D-galactopyranoside), and alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium). The use of enzymatic labels have been described in U.K. 2,019,404, EP 63,879, and by Rotman, Proc. Natl. Acad. Sci., 47, 1981-1991 (1961).

Useful chromophores include, for example, fluorescent, chemiluminescent, and bioluminescent molecules, as well as dyes. Some specific chromophores useful in the present invention include, for example, fluorescein, rhodamine, Texas red, phycoerythrin, umbelliferone, luminol.

The labels may be conjugated to the probe by methods

that are well known in the art. The labels may be directly attached through a functional group on the probe. The probe either contains or can be caused to contain such a functional group. Some examples of suitable functional groups include, for example, amino, carboxyl, sulfhydryl, maleimide, isocyanate, isothiocyanate.

The label may also be conjugated to the probe by attaching a ligand to the probe by a method described above and incubating the conjugate with a labelled receptor for that ligand. Any of the known ligand-receptor combinations is suitable. The biotin-avidin combination is preferred.

For use in immunoassays, the chimeric polypeptides comprising fragments present on P.IA or P.IB described above are used. Equivalent fragments may also be used. Equivalent fragments include substitution, addition and deletion mutations that do not destroy the ability of the polypeptides to detect specific antibodies.

Use of chimeric polypeptides in Vaccines

Since the chimeric polypeptides of the present invention are important for a vital function of *N. gonorrhoeae* and are found in outer membranes, the polypeptides are useful in vaccines for the prevention of diseases caused by *gonococcal* infections, such as gonorrhea. For this purpose, it is necessary for the polypeptide to produce neutralizing antibodies. Neutralizing antibodies are antibodies that significantly inhibit the growth of and/or kill gonococcal cells in vitro or in vivo. Growth of gonococcal cells is significantly inhibited in vivo if the inhibition is sufficient to prevent or reduce the symptoms of the

disease of an infected mammal.

If a polypeptide defines the desired epitopes, but is insufficiently antigenic, it may be conjugated to a carrier molecule to increase antigenicity or half life. Some suitable carrier molecules include keyhole limpet hemocyanin, Ig sequences, TrpE and human or bovine serum albumin. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragment with a cysteine residue on the carrier molecule. Alternatively, the carrier molecule may be joined to the polypeptides of the invention by recombinant means, such as those described above. Antigens may also be cross-linked to self to form polymeric antigens or concatamers.

In addition, delivery of the chimeric porin fragments may be effected by means of incorporation into pilin or flagellin sequences as in the prototypical Salmonella delivery system (B. Stocker, Vaccine Vol. 6 (1988)). Expression by means of vaccinia virus vehicles or presentation on BCG bacillus vehicles is also possible (WHO Meeting, Geneva, June 1989, Vaccine, Vol. 8 (1990)). In each case, the synthetic peptide sequences are presented more profitably to the immune system because of their covalent expression within and at the surface of a larger molecule.

Vaccines comprising the chimeric polypeptides of the invention may be used to inhibit the growth of, or kill, <u>N. gonorrhoeae</u>. Preferably, the chimeric polypeptides comprise fragments that are present in P.IA or P.IB proteins. The chimeric polypeptides may also comprise equivalent fragments. Equivalent fragments for this purpose include substitution, addition or deletion

mutations that produce neutralizing antibodies in a mammalian host such as in a human host.

The present invention further includes vaccine compositions for immunizing mammals, including humans, against infection by N. gonorrhoeae. The vaccines comprise the chimeric polypeptides of the invention or their equivalents and pharmaceutically acceptable media and adjuvants. Equivalents of the chimeric polypeptides are as described above.

The vaccine comprises the antigen in a pharmaceutically acceptable medium. The vaccine may include adjuvants, such as muramyl peptides, and lymphokines, such as interferon, interleukin-1 and interleukin-6. The antigen may be adsorbed on suitable particles, such as aluminum oxide particles, or encapsulated in liposomes, as is known in the art.

Since N. gonorrhoeae infects mucosal linings, it is preferable for the vaccine to present the antigen in a way that maximizes the induction of antibodies of the IgA class. The induction of IgA antibodies may be maximized by exposing the antigen to the gut. Therefore, vaccines that expose the chimeric protein of the invention to the gut, such as oral vaccines, are preferred.

In addition, the antigen may be exposed to the gut by presenting the antigen in a liposome or in a viral or bacterial replicating vehicle. Some bacterial replicating vehicles include, for example, salmonella and shigella. Some examples of viral replicating systems include, vaccinia and adenovirus. Alternatively, the antigen may be presented fused to a protein that has an affinity for the gut. Examples of such proteins include, for example,

adeno spurs and hepatitis B core antigen.

The invention further includes methods of immunizing host mammals, including humans, with an effective amount of the vaccine compositions described above. The vaccine may be administered to a mammal by methods known in the art. Such methods include, for example, oral, intravenous, intraperitoneal, subcutaneous, or intramuscular administration.

EXAMPLES

EXAMPLE 1

A. Synthesis of Polypeptides

Oligonucleotide chains were specifically synthesized on a Model 381A Applied Biosystems apparatus utilizing beta-cyanoethyl phosphoramidites as substrate. Synthesized nucleotide oligomers were deprotected and cleaved from resin supports using standard procedures as recommended by the manufacturer. One may utilize any of a variety of oligonucleotide purification cartridges or proceed with HPLC purification and isolation.

Efficient chain extension is possible to obtain the desired oligonucleotides in length of up to 100 bases. Specific hydrogen-bonding complements of these chains may be also synthesized in the proper polarity. Specific terminal restriction enzyme site compatible ends may also be designed to facilitate annealing and cloning by ligation to vectors or other synthetic duplexes.

Approximately 100 ng of a specific oligonucleotide

chain is annealed to its complement by heating to 100°C briefly and allowing to cool slowly to room temperature in a buffer containing 10 mM TRIS HCl pH 7.5, 0.1 mM EDTA.

The 5'OH termini that result from synthesis and deprotection cleavage may be phosphorylated with polynucleotide kinase enzyme and rATP by any of several well known means (see Maniatis et. al., DNA Cloning Manual). Ligation of specific pairs of oligonucleotide duplexes is accomplished by means of restriction enzyme site termini or "sticky-ends" through specifically designed "overhangs" resulting in compatible hydrogen bonded overlaps. The covalent gap 5'-3' bond may be closed by means of DNA ligase enzyme from E.coli or bacteriophage in simple buffers. In the case of the latter, such as 10 mMTris.HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1mM rATP at 16° incubation for several hours are suitable.

Where large sequences need to be created, pairs or groups of hydrogen bonded duplexes may be mixed and allowed to form a specifically ordered linear structure of total length equal to the length of the individual synthetic duplexes. These are subsequently ligated.

By employing a specific vector or expression vector with suitable compatible restriction site termini, the assembled oligonucleotide structure or mini-gene may be easily and directly cloned and expressed as protein.

An example is given below wherein six individual oligonucleotide chains are mixed and annealed. These are allowed to form a specifically ordered structure which is then ligated and translated as protein information. A

high proportion of the recombinant clones thereby generated contain a properly ordered insert segment. Hybridization to specific, individual oligonucleotides labeled with a reporter group may be used to identify clones. The use of asymmetric enzyme termini (two different restriction enzymes) in the vector allows specific directional, in-frame cloning of similar compatible asymmetric termini from the arms of the assembled structure.

This process can be extended to include more members of synthetic sequences to produce larger specific coding arrays. Alternatively, an intermediate structure may be cloned, isolated and used as a substrate for further expansion of synthetic sequences by means of specific restriction enzyme sites previously applied within the coding domain. In this way, a given sequence may be expanded, contracted or otherwise permuted in a directed manner. Specifically, chimeric arrays may be produced and easily analyzed.

In order to express specific polypeptides representing epitopes at interesting protein domains, a controllable protein expression system is employed. These systems may involve the juxtaposition of a promoter to control the amino acid coding sequence as a non-fusion process or may involve the linkage of the chimeric sequences to an existing protein coding sequence that itself is under the regulated control of a plasmid. This is known as a fusion protein system. The non-fusion system can utilize any of several well-known, characterized and available promoters such as <u>trp</u>, <u>trc</u>, <u>tic</u>, <u>tac</u>, <u>lac</u>, P_L, etc. Fusion protein systems can involve linkage of chimeric coding sequences to trpE, β-

galactosidase, Protein A, maltose binding protein, etc.

As a representative general example, synthetic *N. gonorrhoeae* Porin sequences chosen from specific domains of Porin P.IA and Porin P.IB strain sequences are chosen. These amino acid sequences, otherwise relatively separated in nature, are converted into *E. coli* biased codons and chemically synthesized. Multiple oligonucleotide chains may be required to efficiently span the chosen sets of sequences. These are synthesized in such a manner that assembly by annealing is ordered through internal compatible or "sticky" ends. The extreme outside termini may be designed so as to provide or make available termini specific for various restriction enzymes.

A plasmid that expresses a chimeric polypeptide containing fragments 2 and 6 is called pGC26. To make pGC26, the vector pATH20 is digested at its cloning linker by the enzymes EcoRI and HindIII. PATH20 is a member of the PATH vector systems described by Dieckmann and Tzagoloff in the Journal of Biological Chemistry 260, 1513-120 (1985). The nucleotide sequence of the polylinker in PATH20 is shown in Figure 2. This polylinker, embedded in the anthranilate synthetase gene or trpE product, makes possible insertion of foreign amino acid coding sequences as "read-through" fusion proteins or chimeric polypeptides. If the proper reading frame triplet codon pattern is identified, the EcoRI site of the vector trpE protein may be joined to the EcoRI site of the synthesized chimeric oligonucelotide. Similarly, the respective HindIII site sequences of the nucleic acids may be annealed and ligated. In the case of pGC26, the six oligonucleotides a-f (see Figure 3A) are prepared and ligated to each other and to PATH20 at the EcoRI and HindIII sites. The relationship of fragments a-f to each

other and to the EcoRI and HindIII sites of PATH 20 is shown in Figure 3B. The resulting plasmid expresses the trpE protein under the IAA regulated control of the trp promoter and co-expresses the N. gonorrhoeae Porin A/B sequences (fragments 2 and 6) as a co-linear open reading frame carboxy-extension.

This new plasmid, pGC26, expresses a novel protein of molecular weight larger than the native sequence by an increment of N. gonorrhoeae sequence size. Immunologic techniques may be employed to locate and identify individual N. gonorrhoeae epitopes and their co-expression with other distinct N. gonorrhoeae epitopes. In the case of the Porins, class A and class B sequences may be localized to a single unique protein sequence. In this way, specific combinations of sequences or potential epitopes may be obtained with precision and in useful yield by reproducible methods. Standard techniques may be employed to determine if these proteins are capable of eliciting specific antibody responses, either B or T cell mediated. These antisera would be expected to represent specific responses, in the same animal, to both or all portions or epitopes of the chimeric sequence. These antisera would represent responses to individual epitopes not usually experienced in nature in that particular intertypic combination or display. These antisera may be easily examined to determine whether or not they possess the ability to neutralize or inactivate the parental N. gonorrhoeae organisms representative of the individual chimeric epitopes (e.g., Porin A and/or B).

In a similar manner, a hybrid chimeric polypeptide containing a central PIB fragment flanking two PIA amino and carboxy sequences may be constructed from fragments 2, 6 and 4. In the PIA/PIB expressions clone pGC26 (see

above), a synthetic chimeric porin sequence is cloned into the pATH20 polylinker following the trpE sequence at EcoRI and HindIII sites in phase (see Figure 3B). A unique <u>Bsm</u>I recognition sequence is present in oligonucleotide fragment c (Figure 3A), which represents the carboxy end of the oligonucleotide encoding PIB fragment 6 (Figure 1A). Digestion of the original pGC26 plasmid by the restriction enzymes <u>Bsm</u>I and <u>Hind</u>III allows additional sequences to be covalently joined, in frame, to existing 2/6 synthetic sequences as extensions.

Thus, amino acid sequence 4 (Figure 1A) starting with the amino acid R at position 178 may be added to the carboxy end of GC26 by conjugating pGC26 with <u>Bsm</u>I and <u>Hind</u>III in the presence of oligonucleotides g-j (Figure 4A). The oligonucleotides (g) 5' CATTCCGAGCCTGTTTGTTTTCGTTCAGTACGCTGGTTTCTAC 3' and (h) 5' ACGTTTGTAGAAACCAGCGTACTGAACGAAAACAAACAFGCTCGGAATGCT 3', anneal and ligate as above to the oligonucleotide pair (i) 5' AAACCTCACTCCTACACCACCGAAAAACACCAGGTTCACCGTCTGGTTGGTTA 3' and (j) 5' AGCTTAACCAACCAGACGGTGAACCTGGTCTTTTTCGGTGGTGTAGGAGTG 3', as well as to pGC26. The relationship of oligonucleotides g-j to each other and to the <u>Bsm</u>I and <u>Hind</u>III sites of pGC26 is given schematically in Figure 4B.

This new clone, when grown and suitably induced to express fusion protein, generates a chimeric polypeptide that is 25 amino acids longer than the pGC26 product described above. This new protein is identifiable by

specific reastion in ELISA and western blot with polyclonal anti-N. gonorrhoeae sera or monoclonal anti-peptide sera specific for porin A or B regions 2, 6, 4.

Essentially, methods such as described above for the models pGC26 and pGC264 may serve as a means of identifying pertinent cross-serotypic vaccines. All relevant serovars of an otherwise mutually exclusive family of related organisms may thus be tested for neutralization with this novel immunogen. In nature, where a variety of equally infectious but distinct serovars exist, infection may be more efficiently or broadly prevented.

It thereby becomes possible to choose or identify specific amino acid sequences and produce them via synthetic DNA techniques. It is also evident that specific combinations of amino acid sequences may be co-linearly joined and co-expressed as a single complex polypeptide. In this way, sequences from both related and unrelated species or donors may be juxtaposed in a chimeric array. Sequences from related or unrelated serotypes that do not otherwise exchange genetic information at specific loci or "serovaric" domains may be merged or chimerized (eq A + B = AB or A + B + A = ABA). Where genetic exchange or intertypic exchange is infrequent or not easily detected, specific novel chimeric

polypeptides may be created to produce single entities expressing significant epitopes of both strains. This specific directed process may create molecules of immunologic, vaccine or protective significance not easily obtained reproducibly or with high accuracy or specificity in nature. This is particularly significant in the case of the Porin serotypes A and B which, taken together, are believed to represent all varieties of pathogenic N. gonorrhoeae, but do not otherwise exist in nature.

Although examples may be found wherein strains in nature, otherwise mutually exclusive, may exchange genetic material, this process is rare and not predictable. The identification and creation of specific sets of sequences from each or several organisms allows specific chimeric epitopes to be created and rapidly tested for clinical significance and utilization as a prophylactic or protective vaccine.

B. Construction of the pGC26 plasmid

The expression plasmid PATH20 (see above) was grown in shake flasks containing Luria Broth and 50 micrograms/ml Ampicillin Sulfate. Supercoiled, covalently closed circular plasmid DNA was isolated by CSCI gradient ultracentrifugation. Approximately 10 micrograms of

plasmid was digested to completion with the restriction enzymes EcoRI and HindIII, each of which cleave the vector once. Such cleavage interrupts the TrpE gene (anthranilate synthetase) coding region and creates "sticky end" termini which may be used to accept heterologous DNA sequences, in reading frame, and create chimeric nucleotides following ligation and selection. The enzymes EcoRI and HindIII were added to a 100ul reaction volume, at 10 units each, containing 50mMTris-HCl pH7.5, 10mM mg Cl_2, 50mM NaCl, 10ug plasmid. The reaction was allowed to proceed for 4 hours at which time digestion was verified by gel electrophoresis. The double digested plasmid DNA was isolated by electroelution from 1% agarose gels and ethanol precipitated. Approximately 1ug of this plasmid was combined with 200ng of each of complementary pair of annealled oligonucleotides a/f, b/e and c/d (Figure 3A). Following ligation, the *N. gonorrhoeae* porin epitope sequences a-f, bounded at their extreme termini by EcoRI and HindIII sites, are produced as shown in Figure 5. The solution, 100ul, was brought to 0.3M with NaOAC from a 3M stock and precipitated with ethanol at 0°C for 1 hour. Following centrifugation in an Eppendorf micro-fuge, the DNA pellet was dried in a Savant Speed-Vac under reduced pressure. The pellet was resuspended in 17 ul water and 2ul 10X Ligase buffer (60mMTris pH7.6, 66mM Mg Cl_2, 0.2M DTT) with a final ATP concentration of 1mM. T4

induced DNA ligase (1ul) was added and the reaction incubated at 16C for 12 hours.

To produce ampicillin resistant HB101 colonies bearing recombinant plasmids, 100ul competent E.coli cells were mixed, on ice, with 5ul of the ligated mixture. The cells and DNA were held, on ice, for 30 minutes, pulsed at 42°C for 2 minutes and shaken at 37°C for 30 minutes with 1ml Luria Broth without antibiotics. An aliquot (100ul) was spread onto ampicillin agar plates and allowed to incubate at 32°C overnight. Colonies produced were hybridized versus P_{32} end-labeled oligonucleotides representing the chimeric or individual porin "epitope" sequences of interest. In this way, colonies that represented successful ligation events were rapidly chosen and expanded for analysis.

Aliquots of induced and non-induced (in this case IAA) colony growths, as well as negative control host E. coli were lysed and examined by Coumassie blue gel staining and Western blot versus specific porin or carrier protein (TrpE) antisera. Finally, recombinant chimeric epitope expressors were fully sequenced to verify their integrity.

In order to express protein produced by the

recombinant porin constructions, cultures bearing plasmids were grown to Abs. 600nm of approximately 0.2 - 0.4 in M9 media containing 50ug/ug ampicillin. Indole acrylic acid (IAA) was added (10mg/liter) and the culture shaken at least 5 hours or grown overnight for 8 - 16 hours. Cells were pelleted and resuspended in 25ml TEN buffer (50mMTris7.5, 0.5mMEDTA, 0.3M NaCl). Lysozyme was added to 0.1mg/ml. The protease inhibitors PMSF and Aprotinin were added to 1mM and 10ug/ml respectively. NP-40 was added to 0.2% final concentration and the lysate held at 0°C for 10 minutes. When the viscosity had increased, MgCl2 was added to 10mM and DNAase 1 added to 1ug/ml. When the viscosity had decreased, the suspension was centrifuged at 4000xg for 15 minutes at 4°C and the supernatant discarded. The insoluble pellet was washed 1X with cold TEN and recentrifuged to recover the pellet. This material is highly enriched with recombinant fusion protein and is very suitable for gel electrophoresis, ELISA, Western blot or other immunologic analysis such as animal injection. Recombinant products may be detected by means of specific reactivity of the carrier and/or the ligand sequence by any of the above methods.

The nucleotide sequence obtained as well as the corresponding open reading frame is shown in Figure 4. This sequence has an enterokinase cleavage site after the

first lysine residue (amino acid 7). GC26, which is a chimera of fragments 2 and 6, results from enterokinase cleavage. Enterokinase may be obtained commercially from Sigma Chemical Company, St. Louis Missouri. Methods of Cleaving proteins with enterokinase are known in the art. For example, one may use the procedure recommended by the manufacturer or by Hopp et al., Biotechnology 6, 1204-1210 (1988).

Other recombinants are produced and examined by this process and utilized in vaccine candidate analysis procedures.

EXAMPLE 2

Production of anti-GC26 antisera

Mice are hyperimmunized with GC-30 to show the efficacy of the chimeric polypeptide to induce an anti-P.IA and anti-P.IB humoral response. Bacterial cells containing the construct are washed and lysed in a buffer containing Triton X-100 and sodium deoxycholate. The protein concentration is determined and 100 mg of the chimeric polypeptide or PATH vector alone is injected into female Balb/C mice (8-10 weeks old). The initial injection mixture contains complete Freund's adjuvant.

The animals receive a second injection of 100mg at 7 days with incomplete Freund's adjuvant and a final injection of 100 mg at day 21. Seven days after the last injection, the animals are bled from the retro-orbital socket of the eye, and the sera isolated by centrifugation.

The titer of the anti-P.IA and P.IB humoral response is determined by ELISA. Microtiter plates (96 well) are coated with purified P.IA or P.IB and blocked with 10% new born cell serum in phosphate buffered saline, pH 7.2(NB-10). The sera is serially diluted in NB-10 and added to the plates. After a two hour incubation at 37°C, the plates are washed with saline and probed with goat anti-mouse Ig conjugated to horseradish peroxidase. Following a one hour incubation at 37°C, the plates are washed and binding determined by the addition of a suitable chromogen. The color intensity is determined in an ELISA plate reader at the appropriate wavelength.

Alternatively, the production of anti-GC26 antisera could be accomplished by immunizing mice with affinity purified Trp-GC-26. This can be done using an immuno-affinity column using an anti-TrpE monoclonal antibody to bind the construct. The polypeptide can be diluted from the column using a glycine buffer, pH 2.5. The purified material is used to immunize mice as described above.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ImClone Systems Incorporated

(ii) TITLE OF INVENTION: Recombinant Hybrid Porin Epitopes

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: ImClone Systems Incorporated

(B) STREET: 180 Varick Street

(C) CITY: New York

(D) STATE: New York

(E) COUNTRY: US

(F) ZIP: 10014

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 13-MAR-1992

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Feit, Irving N.

(B) REGISTRATION NUMBER: 28,601

(C) REFERENCE/DOCKET NUMBER: GOL-1T

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 212-645-1405

(B) TELEFAX: 212-645-2054

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Neisseria gonorrheae

(B) STRAIN: FA19

(vii) IMMEDIATE SOURCE:

(B) CLONE: P.IA fragment 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Val Thr Leu Tyr Gly Thr Ile Lys Ala Gly Val Glu Thr Ser Arg
1 5 10 15

Ser Val Ala His His Gly Ala Gln Ala Asp
20 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Neisseria gonorrheae

(B) STRAIN: FA19

(vii) IMMEDIATE SOURCE:

(B) CLONE: P.IA fragment 2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Val Glu Thr Ser Arg Ser Val Ala His His Gly Ala Gln Ala Asp Arg
1               5                   10                  15

Val Lys Thr Ala Thr Glu Ile Ala Asp
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Neisseria gonorrheae

(B) STRAIN: FA19

(vii) IMMEDIATE SOURCE:

(B) CLONE: P.IA fragment 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Thr Gly Gly Phe Asn Pro Trp Glu Gly Lys Ser Tyr Tyr Leu Gly
1 5 10 15

Leu Ser Asn Ile Ala Gln Pro Glu Glu Arg His Val
20 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Neisseria gonorrheae

(B) STRAIN: FA19

(vii) IMMEDIATE SOURCE:

(B) CLONE: P.IA fragment 4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Phe Val Gln Tyr Ala Gly Phe Tyr Lys Arg His Ser Tyr Thr Thr Glu
1 5 10 15

Lys His Gln Val His Arg Leu Val Gly
20 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Neisseria gonorrheae

(B) STRAIN: MS11

(vii) IMMEDIATE SOURCE:

(B) CLONE: P.IB fragment 5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Ala Ile Lys Ala Gly Val Gln Thr Tyr Arg Ser Val Glu His Thr
1 5 10 15

Asp Gly Lys Val Ser Lys Val Glu Thr Gly Ser
20 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Neisseria gonorrheae

(B) STRAIN: MS11

(vii) IMMEDIATE SOURCE:

(B) CLONE: P.IA fragment 6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Leu Phe Gln Arg Tyr Gly Glu Gly Thr Lys Lys Ile Glu Tyr Glu
1 5 10 15

His Gln Val Tyr Ser Ile Pro Ser Leu Phe Val
20 25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 78 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: PATH20 POLYLINKER

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATTGAGATCC CCCCGAATTG GGAATTCGAG CTCGGTACCC GGGGATCCTC TAGAGTCGAC 60

CTGCAGGCAT GCAAGCTT 78

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 181 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: GC26 SYNTHETIC DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATTCGATGA TGACGATAAA GTGGAAACCT CCCGCTCCGT GGCGCACCAT GGCGCGCAGG 60

CGGATCGCGT TAAAACCGCG ACCGAAATTG CGGATCTGGG CCTGTTCCAG CGCTACGGCG 120

AAGGCACCAA AAAAATTGAA TACGAACATC AGGTTTATAG CATTCCGAGC CTGTTTGTTT 180

A 181

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 182 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: GC26 SYNTHETIC DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGCTTAAACA AACAGGCTCG GAATGCTATA AACCTGATGT TCGTATTCAA TTTTTTTGGT 60

GCCTTCGCCG TAGCGCTGGA ACAGGCCCAG ATCCGCAATT TCGGTCGCGG TTTTAACGCG 120

ATCCGCCTGC GCGCCATGGT GCGCCACGGA GCGGGAGGTT TCCACTTTAT CGTCATCATG 180

CG 182

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: GC264 SYNTHETIC DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CATTCCGAGC CTGTTTGTTT TCGTTCAGTA CGCTGGTTTC TAC 43

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: GC264 SYNTHETIC DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACGTTTGTAG AAACCAGCGT ACTGAACGAA AACAAACACG CTCGGAATGC T 51

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: GC264 SYNTHETIC DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAACCTCACT CCTACACCAC CGAAAAACAC CAGGTTCACC GTCTGGTTGG TTA 53

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: GC264 SYNTHETIC DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCTTAACCA ACCAGACGGT GAACCTGGTC TTTTTCGGTG GTGTAGGAGT G 51

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 182 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: GC26 SYNTHETIC DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AATTCGATGA TGACGATAAA GTAGAAACTT CCCGCTCCGT AGCTCACCAT GGAGCTCAGG 60

CGGATCGCGT TAAAACCGCT ACCGAAATCG CTGATTTGGG CTTGTTCCAA AGATACGGCG 120

AAGGCACTAA AAAAATCGAA TACGAACATC AAGTTTATAG TATCCCAGCC TGTTTGTTTA 180

AA 182

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 183 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: GC26 SYNTHETIC DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGCTTTTAAA CAAACAGGCT GGGGATACTA TAAACTTGAT GTTCGTATTC GATTTTTTTA 60

GTGCCTTCGC CGTATCTTTG GAACAAGCCC AAATCAGCGA TTTCGGTAGC GGTTTTAACG 120

CGATCCGCCT GAGCTCCATG GTGAGCTACG GAGCGGGAAG TTTCTACTTT ATCGTCATCA 180

TCG 183

CLAIMS

1. A polypeptide that is non-toxic in E. coli wherein the polypeptide comprises at least one antigenic sequence present in P.IA of N. gonorrhoeae and at least one antigenic sequence present in P.IB of N. gonorrhoeae.

2. A polypeptide according to claim 1 wherein the antigenic sequence present in P.IA of N. gonorrhoeae is selected from fragments 1-4 of Figure 1A.

3. A polypeptide according to claim 1 wherein the antigenic sequence present in P.IB of N. gonorrhoeae is selected from fragments 5 and 6 of Figure 1A.

4. A polypeptide according to claim 1 wherein the polypeptide is fused to a carrier peptide.

5. A polypeptide according claim 4 wherein the carrier peptide is separated from the polypeptide by a cleavable site.

6. A polypeptide according to claim 1 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 125 amino acid residues.

7. A polypeptide according to claim 1 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 75 amino acid residues.

8. A polypeptide according to claim 1 wherein the antigenic sequence or antigenic sequences present in

P.IA of N. gonorrhoeae consist of no more than 50 amino acid residues.

9. A polypeptide according to claim 1 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 125 amino acid residues.

10. A polypeptide according to claim 1 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 75 amino acid residues.

11. A polypeptide according to claim 1 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 50 amino acid residues.

12. A polypeptide according to claim 9 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 125 amino acid residues.

13. A polypeptide according to claim 10 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 75 amino acid residues.

14. A polypeptide according to claim 11 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 50 amino acid residues.

15. A polypeptide comprising at least one antigenic sequence present in P.IA of N. gonorrhoeae and at

least one antigenic sequence present in P.IB of N. gonorrhoeae, the total number of amino acids in the antigenic sequence present in P.IA being no more than 125 and the total number of amino acids in the antigenic sequence present in P.IB being no more than 125.

16. A polypeptide according to claim 15 wherein the antigenic sequence present in P.IA of N. gonorrhoeae is selected from fragments 1-4 of Figure 1A.

17. A polypeptide according to claim 15 wherein the antigenic sequence present in P.IB of N. gonorrhoeae is selected from fragments 5 and 6 of Figure 1A.

18. A polypeptide according to claim 15 wherein the polypeptide is fused to a carrier peptide.

19. A polypeptide according to claim 18 wherein the carrier peptide is separated from the polypeptide by a cleavable site.

20. A polypeptide according to claim 15 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 75 amino acid residues.

21. A polypeptide according to claim 15 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 50 amino acid residues.

22. A polypeptide according to claim 15 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 75

amino acid residues.

23. A polypeptide according to claim 15 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 50 amino acid residues.

24. A polypeptide according to claim 20 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 75 amino acid residues.

25. A polypeptide according to claim 21 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 50 amino acid residues.

26. A method for detecting the presence of antibodies specific for P.IA and, simultaneously, antibodies specific for P.IB of N. gonorrhoeae in a sample comprising the steps of:

(a) incubating the sample with a chimeric polypeptide according to claim 1 or claim 15; and
(b) detecting the presence of the antibody bound to the chimeric polypeptide.

27. A method of immunizing a mammal simultaneously against N. gonorrhoeae serovars IA and IB comprising administering to the mammal an effective amount of a chimeric polypeptide according to claim 1 or claim 15.

28. A vaccine composition comprising an effective amount

of a chimeric polypeptide according to claim 1 or claim 15 in a pharmaceutically acceptable medium.

29. A DNA molecule that encodes a polypeptide that is non-toxic in E. coli wherein the polypeptide comprises at least one antigenic sequence present in P.IA of N. gonorrhoeae and at least one antigenic sequence present in P.IB of N. gonorrhoeae.

30 A DNA molecule according to claim 29 wherein the antigenic sequence present in P.IA of N. gonorrhoeae is selected from fragments 1-4 of Figure 1A.

31. A DNA molecule according to claim 1 wherein the antigenic sequence present in P.IB of N. gonorrhoeae is selected from fragments 5 and 6 of Figure 1A.

32. A DNA molecule according to claim 29 wherein the polypeptide is fused to a carrier peptide.

33. A DNA molecule according to claim 32 wherein the carrier peptide is separated from the polypeptide by a cleavable site.

34. A DNA molecule according to claim 29 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 125 amino acid residues.

35. A DNA molecule according to claim 29 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 75 amino acid residues.

36. A DNA molecule according to claim 29 wherein the

antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 50 amino acid residues.

37. A DNA molecule according to claim 29 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 125 amino acid residues.

38. A DNA molecule according to claim 29 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 75 amino acid residues.

39. A DNA molecule according to claim 29 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 50 amino acid residues.

40. A DNA molecule according to claim 37 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 125 amino acid residues.

41. A DNA molecule according to claim 38 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 75 amino acid residues.

42. A DNA molecule according to claim 39 wherein the antigenic sequence or antigenic sequences present in P.IA of N. gonorrhoeae consist of no more than 50 amino acid residues.

43. A DNA molecule that encodes a polypeptide comprising

at least one antigenic sequence present in P.IA of _N. gonorrhoeae_ and at least one antigenic sequence present in P.IB of _N. gonorrhoeae_, the total number of amino acids in the antigenic sequence present in P.IA being no more than 125 and the total number of amino acids in the antigenic sequence present in P.IB being no more than 125.

44. A DNA molecule according to claim 43 wherein the antigenic sequence present in P.IA of _N. gonorrhoeae_ is selected from fragments 1-4 of Figure 1A.

45. A DNA molecule according to claim 43 wherein the antigenic sequence present in P.IB of _N. gonorrhoeae_ is selected from fragments 5 and 6 of Figure 1A.

46. A DNA molecule according to claim 43 wherein the polypeptide is fused to a carrier peptide.

47. A DNA molecule according to claim 46 wherein the carrier peptide is separated from the polypeptide by a cleavable site.

48. A DNA molecule according to claim 43 wherein the antigenic sequence or antigenic sequences present in P.IA of _N. gonorrhoeae_ consist of no more than 75 amino acid residues.

49. A DNA molecule according to claim 43 wherein the antigenic sequence or antigenic sequences present in P.IA of _N. gonorrhoeae_ consist of no more than 50 amino acid residues.

50. A DNA molecule according to claim 43 wherein the antigenic sequence or antigenic sequences present in

P.IB of N. gonorrhoeae consist of no more than 75 amino acid residues.

51. A DNA molecule according to claim 15 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 50 amino acid residues.

52. A DNA molecule according to claim 20 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 75 amino acid residues.

53. A DNA molecule according to claim 21 wherein the antigenic sequence or antigenic sequences present in P.IB of N. gonorrhoeae consist of no more than 50 amino acid residues.

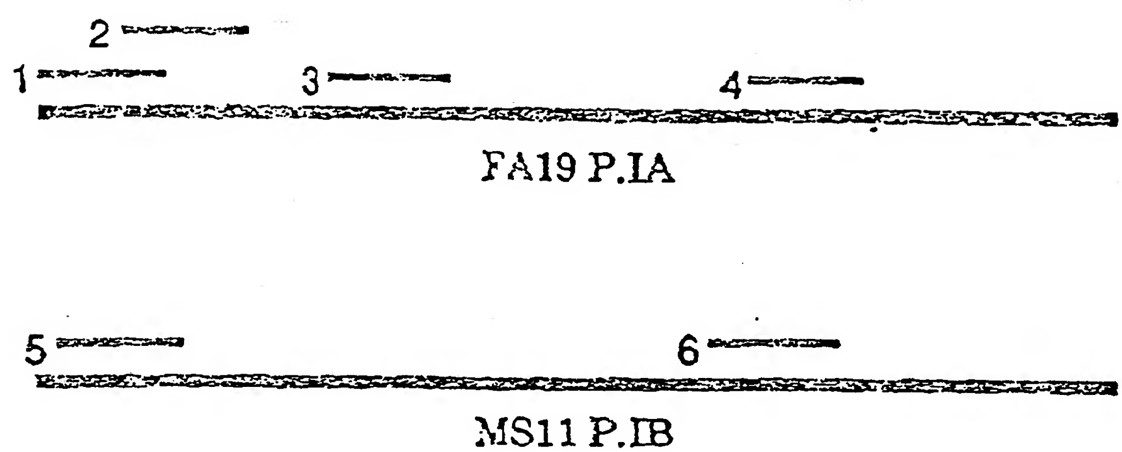
FIGURE 1A

P.IA Fragments		Amino Acids
1.	DVTLYGTIKAGVETSRSVAHHGAQAD	1-26
2.	VETSRSVAHHGAQADRVKTATEIAD	12-36
3.	DTGGFNPWEGKSYYLGLSNIAQPEERHV	99-126
4.	FVQYAGFYKRHSYTTEKHQVHRLVG	169-193

P.IB Fragments		Amino Acids
5.	GAIKAGVQTYRSVEHTDGKVSKVETGS	6-32
6.	GLFQRYGEGTKKIEYEHQVYSIPSLFV	178-204

FIGURE 1B

Approximate relative position of fragments 1-6 on P.IA and P.IB*



* Not to scale

FIGURE 2

ATT GAG ATC CCC CCG AAT TGG GAA TTC GAG CTC GGT ACC CGG GGA TCC TCT ACA GTC GAC CTG CAG GCA TGC AAG CTT

FIGURE 3A

```
 a
          10         20         30         40         50
 AATTCGATG ATGACGATAA AGTGGAAACC TCCCGCTCCG TGGCGCACCA
     GCTAC TACTGCTATT TCACCTTTGG AGGGCGAGGC ACCGCGTGGT

                b
        60         70         80         90        100
TGGCGCGCAG GCGGATCGCG TTAAAACCGC GACCGAAATT GCGGATCTGG
ACCGCGCGTC CGCCTAGCGC AATTTTGGCG CTGGCTTTAA CGCCTAGACC
                   f              c
       110        120        130        140        150
GCCTGTTCCA GCGCTACGGC GAAGGCACCA AAAAAATTGA ATACGAACAT
CGGACAAGGT CGCGATGCCG CTTCCGTGGT TTTTTTAACT TATGCTTGTA
                             e
       160        170        180
CAGGTTTATA GCATTCCGAG CCTGTTTGTT TA
GTCCAAATAT CGTAAGGCTC GGACAAACAA ATTCGA
                             d
```

FIGURE 3B

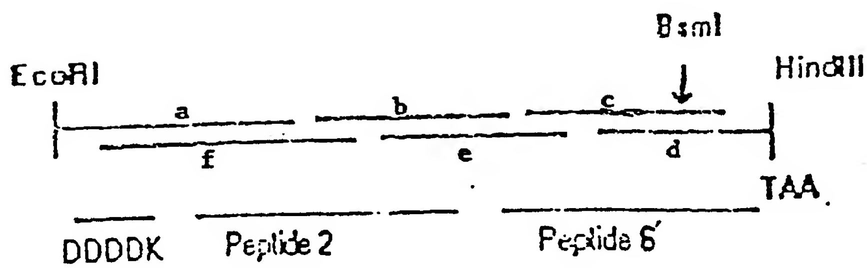


FIGURE 4A

(g) 5′ CATTCCGAGCCTGTTTGTTTTCGTTCAGTACGCTGGTTTCTAC 3′

(h) 5′ ACGTTTGTAGAAACCAGCGTACTGAACGAAAACAAACACGCTCGGAATGCT 3′

(i) 5′ AAACCTCACTCCTACACCACCGAAAAACACCAGGTTCACCGTCTGGTTGGTTA 3′

(j) 5′ AGCTTAACCAACCAGACGGTGAACCTGGTCTTTTTCGGTGGTGTAGGAGTG 3′.

FIGURE 4B

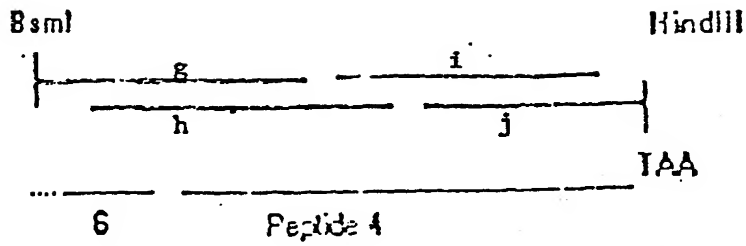


FIGURE 5

```
         10         20         30         40         50
AATTCGATG ATGACGATAA AGTAGAAACT TCCCGCTCCG TAGCTCACCA
    GCTAC TACTGCTATT TCATCTTTGA AGGGCGAGGC ATCGAGTGGT

        60         70         80         90        100
TGGAGCTCAG GCGGATCGCG TTAAAACCGC TACCGAAATC GCTGATTTGG
ACCTCGAGTC CGCCTAGCGC AATTTTGGCG ATGGCTTTAG CGACTAAACC

       110        120        130        140        150
GCTTGTTCCA AAGATACGGC GAAGGCACTA AAAAAATCGA ATACGAACAT
CGAACAAGGT TTCTATGCCG CTTCCGTGAT TTTTTTAGCT TATGCTTGTA

       160        170        180
CAAGTTTATA GTATCCCCAG CCTGTTTGTT TAAA
GTTCAAATAT CATAGGGGTC GGACAAACAA ATTTTCGA
```

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02090

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12P 21/02; C12N 15/00, 1/21; A61K 37/02
US CL : 435/69.1, 172.3, 252.33, 320.1; 530/300

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/69.1, 172.3, 252.33, 320.1; 530/300

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[6]

CAS ONLINE, search terms: Neisseria gonorrhoeae, expression, Escherichia coli

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Proceedings of the National Academy of Sciences, Volume 85, issued September 1988, Carbonetti et al., "Genetics of Protein I of Neisseria gonorrhoeae: Construction of Hybrid Porins", pages 6841-6845, see the abstract and Figure 3.	1-53
Y	Proceedings of the National Academy of Sciences, Volume 84, issued December 1987, N.H. Carbonetti et al., "Molecular Cloning and Characterization of the Structural Gene for Protein I, the Major Outer Membrane Protein of Neisseria gonorrhoeae", pages 9084-9088, see the abstract and Figure 3.	1-53
Y	Proceedings of the National Academy of Sciences, Volume 84, issued November 1987, E.C. Gotschlich et al., "Porin Protein of Neisseria gonorrhoeae: Cloning and Gene Structure", pages 8135-8139, see Figure 4.	1-53
Y	Proceedings of the National Academy of Sciences, Volume 78, No. 6, issued June 1981, T.P. Hopp, et al., "Prediction of Protein Antigenic Determinants from Amino Acid Sequences", pages 3824-3828. See the abstract and Figure 1.	1-53

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
08 JUNE 1992	25 JUN 1992
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	JOAN ELLIS

Form PCT/ISA/210 (second sheet)(May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Journal of Molecular Biology, Volume 157, issued 1982, J. Kyte, et al., A Simple Method for Displaying the Hydropathic Character of a Protein", pages 105-132, see the abstract.	1-53
Y	Journal of General Microbiology, Volume 132, issued 1986, J.N. Fletcher, et al., "Monoclonal Antibodies to Gonococcal Outer Membrane Protein I: Location of a Conserved Epitope on Protein IB", see pages 1611-1620.	1-53

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	A.R. Gennaro, "Remington's Pharmaceutical Science", Eighteenth Edition, published 1992 by Mack Publishing Co. (Easton, Pa.) Chapter 72, pages 1389-1404, see entire document.	27, 28